TRANSLATION STATEMENT

Study Title: Chromosomal Aberration Test of [redacted] in Cultured Mammalian Cells

Study Number: SR09243

I, the undersigned, hereby certify that this is a true and accurate translation from the original Japanese final report into English, which was reviewed properly.

Safety Research Institute for Chemical Compounds Co., Ltd.

Translated by:

Kohtaro Kawamura — May 14, 2012

Kohtaro Kawamura, Study Director — Date

FINAL REPORT

(Translation)

Study Title: Chromosomal Aberration Test of in Cultured Mammalian Cells

Study Number: SR09243

Safety Research Institute for Chemical Compounds Co., Ltd.

STATEMENT

Study Title: Chromosomal Aberration Test of in Cultured Mammalian Cells

Study Number: SR09243

1. This study was conducted in compliance with GLP standards "On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.," Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau (PFSB), Ministry of Health, Labour and Welfare, Japan (MHLW), November 21, 2003; No. 3 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, Japan (METI), November 17, 2003; and No. 031121004 of the Environmental Policy Bureau, Ministry of the Environment, Japan (MOE); and "Partial Amendment of 'On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.'," Notification No. 0704001 of the PFSB, MHLW, July 4, 2008; No. 2 of the Manufacturing Industries Bureau, METI, June 30, 2008; and No. 080704001 of the Environmental Policy Bureau, MOE. The test method used in this study was based on "On the Test Method Concerning New Chemical Substances, etc." (Notification No. 1121002 of the PFSB, MHLW, November 21, 2003; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2003; and No. 031121002 of the Environmental Policy Bureau, MOE); and "Partial Amendment of 'On the Test Method Concerning New Chemical Substances, etc.'" (Notification No. 1120001 of the PFSB, MHLW, November 20, 2006; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2006; and No. 061120001 of the Environmental Policy Bureau, MOE)
2. This study was conducted in compliance with the study protocol, and no environmental factors that could have affected the reliability of the study were found.

Safety Research Institute for Chemical Compounds Co., Ltd.

Name and seal affixed in the original	August 10, 2010
Kohtaro Kawamura, Study Director	Date

QUALITY ASSURANCE STATEMENT

Study Title: Chromosomal Aberration Test of in Cultured Mammalian Cells

Study Number: SR09243

This study was inspected by the Quality Assurance Unit of Safety Research Institute for Chemical Compounds Co., Ltd. as follows:

Phase of study inspection	Date of inspection	Date of report to Study Director	Date of report to Management
Study protocol	April 23, 2010	April 23, 2010	April 23, 2010
Study protocol amendment (No. 1)	May 17, 2010	May 17, 2010	May 17, 2010
Study protocol amendment (No. 2)	June 29, 2010	June 29, 2010	June 29, 2010
Receipt, labeling and storage of test substance	April 23, 2010	April 23, 2010	April 23, 2010
Preparation of test substance	May 17, 2010	May 17, 2010	May 17, 2010
Conduct of study	May 17, 2010	May 17, 2010	May 17, 2010
Preparation of specimens	May 20, 2010	May 20, 2010	May 20, 2010
Observation	June 2, 2010	June 2, 2010	June 2, 2010
Raw data	June 25, 2010	June 25, 2010	June 25, 2010
Final report (draft): Tables and Figures	June 25, 2010	June 25, 2010	June 25, 2010
Final report (draft): Text	June 25, 2010	June 25, 2010	June 25, 2010
Final report	August 10, 2010	August 10, 2010	August 10, 2010

1. This study was conducted in compliance with "On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.," Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau (PFSB), Ministry of Health, Labour and Welfare, Japan (MHLW), November 21, 2003; No. 3 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, Japan (METI), November 17, 2003; No. 031121004 of the Environmental Policy Bureau, Ministry of the Environment, Japan (MOE); "Partial Amendment of 'On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.'," Notification No. 0704001 of the PFSB, MHLW, July 4, 2008; No. 2 of the Manufacturing Industries Bureau, METI, June 30, 2008; and No. 080704001 of the Environmental Policy Bureau, MOE; "On the Test Method Concerning New Chemical Substances, etc." (Notification No. 1121002 of the PFSB, MHLW, November 21, 2003; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2003; and No. 031121002 of the Environmental Policy Bureau, MOE); and "Partial Amendment of 'On the Test Method Concerning New Chemical Substances, etc.'" (Notification No. 1120001 of the PFSB, MHLW, November 20, 2006; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2006; and No. 061120001 of the Environmental Policy Bureau, MOE).
2. The Quality Assurance Unit has reviewed the final report and determined the following: this study was conducted in compliance with the study protocol, the methods and procedures of this study were accurately described in this report, and the results presented in this report accurately reflect the raw data generated during this study.

Safety Research Institute for Chemical Compounds Co., Ltd.

Name and seal affixed in the original
Taku Katano, QA Representative

August 10, 2010
Date

CONTENTS

Study title:	Chromosomal Aberration Test of in Cultured Mammalian Cells
Study number:	SR09243
Purpose of the study:	The clastogenic potential of was evaluated *in vitro* using Chinese hamster lung (CHL/IU) cells.

Good Laboratory Practice standards and test guidelines

Good Laboratory Practice (GLP) standards:

"On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.," Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau (PFSB), Ministry of Health, Labour and Welfare, Japan (MHLW), November 21, 2003; No. 3 of the Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, Japan (METI), November 17, 2003; and No. 031121004 of the Environmental Policy Bureau, Ministry of the Environment, Japan (MOE)

"Partial Amendment of 'On the Standard for the Test Facility Conducting Tests Concerning New Chemical Substances, etc.'," Notification No. 0704001 of the PFSB, MHLW, July 4, 2008; No. 2 of the Manufacturing Industries Bureau, METI, June 30, 2008; and No. 080704001 of the Environmental Policy Bureau, MOE

Test guidelines: "On the Test Method Concerning New Chemical Substances, etc." (Notification No. 1121002 of the PFSB, MHLW, November 21, 2003; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2003; and No. 031121002 of the Environmental Policy Bureau, MOE)

"Partial Amendment of 'On the Test Method Concerning New Chemical Substances, etc.'" (Notification No. 1120001 of the PFSB, MHLW, November 20, 2006; No. 2 of the Manufacturing Industries Bureau, METI, November 13, 2006; and No. 061120001 of the Environmental Policy Bureau, MOE)

Study sponsor

Name:

Address:

Study monitor:

Test facility

Name:	Safety Research Institute for Chemical Compounds Co., Ltd.
Address:	363-24 Shin-ei, Kiyota-ku, Sapporo 004-0839, Japan
Management:	Masao Kiguchi

Study Director

Name:	Kohtaro Kawamura
Company and division:	Safety Research Division, Safety Research Institute for Chemical Compounds Co., Ltd.

Study personnel

Test substance management:	Shiho Kodama (responsible person), Misako Ohkubo, and Kohtaro Kawamura
Test operation:	Ikuko Endo, Kohtaro Kawamura, and Wataru Shimatani

Testing period

Study initiation:	April 23, 2010
Receipt of test substance:	January 18, 2010
Start of experiment:	April 29, 2010
Preliminary test	
Initiation of treatment with the test substance:	April 29, 2010
Fixation and staining:	April 30, 2010
Main test	
Initiation of treatment with test substance:	May 17, 2010
Preparation of specimens:	May 18, 2010
Experiment completion (completion of observation):	June 4, 2010
Study completion:	August 10, 2010

SUMMARY

The clastogenic potential of was evaluated *in vitro* using Chinese hamster lung (CHL/IU) cells. The test was performed in three test series: short-term treatment with and without metabolic activation and continuous treatment for 24 h.

In the preliminary test (cell-growth inhibition test: 14.6 to 1870 μg/mL [equivalent to 10 mM]), cell growth was slightly inhibited at the high dose after continuous treatment for 24 h, and no effects were observed in cell growth after short term treatment with or without metabolic activation. Precipitation of the test substance or effects of the test substance treatment on the culture pH were not observed in any test series.

Based on the results of the preliminary test, the highest dose of each test series was set at 1870 μg/mL, and lower doses were set with a twofold dilution series (3 or 4 doses in total) in the main test (chromosomal aberration test). As the results, the incidences of structural and numerical aberrations of chromosomes were less than 5% at all doses of all test series, which showed negative results. No precipitation of the test substance or effects of the test substance treatment on the culture pH were observed in any test series, which were the same as in the preliminary test.

In the positive control group, the incidence of structural aberrations of chromosomes was clearly positive in all test series, demonstrating that the cell line used in this study had sufficient sensitivity.

These results indicate that is not clastogenic in cultured mammalian cells under the conditions used in the present test.

INTRODUCTION

To evaluate the clastogenic potential of *in vitro*, a chromosomal aberration test was performed using Chinese hamster lung (CHL/IU) cells.

MATERIALS AND METHODS

1. Test substance

Name:
Abbreviation:
CAS No.:
Reference Number in Gazetted List in Japan:

Rational formula (structural formula):

Molecular weight:
Physicochemical properties:

Lot No.:

Supplier:	Study sponsor
Amount obtained:	2 containers (NET 1000 g and 1090 g, shared with related tests) Obtained as an aqueous solution (clear liquid) containing solid content 24.0%, impurities 0.85% (to the solid content), and Cl ion 35 ppm (Appendix 1-1).
Stability:	Deliquescent, stable in water After completion of test operation, the analysis results of the test substance were obtained and stability was confirmed (Appendix 1-2).
Storage conditions:	Containers were sealed and stored in a refrigerator (measured temperature: 2 to 8 °C).
Storage location:	The test substance storage room (refrigeration room) and the refrigerator in the mutagenicity test room
Storage period:	January 18 (receipt) to May 17, 2010 (date of final use)
Caution in handling:	Handled in a clean bench wearing gloves, protection

glasses, masks, etc.

Remaining test substance: After completion of test operation including that in the related tests, the remaining test substance was returned to the supplier.

2. Preparation of test substance

The test substance is soluble in water, and was dissolved in and diluted with Japanese Pharmacopoeia water for injection (Lot No. 8L88, Otsuka Pharmaceutical Factory, Inc.) to the prescribed concentrations before use. The prepared concentration was calculated on the basis that the purity of the test substance was 24.0%.

For the preliminary test, an 18.7 mg/mL preparation was prepared, which was serially diluted in a twofold dilution series to prepare 9.35, 4.68, 2.34, 1.17, 0.584, 0.292, and 0.146 mg/mL preparations.

For the main test, an 18.7 mg/mL preparation was prepared, which was serially diluted in a twofold dilution series to prepare 9.35, 4.68, and 2.34 mg/mL preparations.

Regarding the stability, the preparations showed no visible reactivity (such as discoloration, generation of heat, and foaming) to the vehicle at preparation of test substance in either the preliminary or main test.

The test preparations were used within 0.8 h after preparation in both the preliminary and main tests.

The test solutions were prepared in a clean bench using protection glasses, masks, gloves, white lab coat, etc. to avoid inhalation and contact with eyes, skin and clothes. Remaining preparations were collected as industrial waste to be incinerated.

3. Negative control substance

Japanese Pharmacopoeia water for injection (Lot No. 8L88; expiration date, December 2013; Otsuka Pharmaceutical Factory, Inc.) was used as the negative control substance, without any further preparation.

The negative control substance was added at a concentration of 10% (vol) to the culture medium in each plate.

4. Positive control substances

Mitomycin C (Lot No. 498AFJ; expiration date, October 2010; Kyowa Hakko Kogyo Co., Ltd.) was used as the positive control substance for treatment without metabolic activation. The compound was stored at room temperature and dissolved in Japanese Pharmacopoeia water for injection (Lot No. 7K81, Otsuka Pharmaceutical Factory, Inc.) to obtain concentrations of 5 and 10 μg/mL. One vial of mitomycin C contained 2 mg (potency) of Japanese Pharmacopoeia mitomycin C; hence, calculations for preparation were made on the basis that 1 mg (potency) of mitomycin C was equivalent to 1 mg of mitomycin C.

3,4-benzopyrene (Lot No. 8JB8G; expiration date, July 2014 [for 5 years after purchase]; Tokyo Chemical Industry Co., Ltd.) was used as the positive control substance for treatment with metabolic activation. The compound was stored in a cool place (2 to 8 °C) and dissolved in dimethyl sulfoxide (Lot No. TA026, Dojindo Laboratories) to obtain a concentration of 1 mg/mL. The content of 3,4-benzopyrene in the product purchased was 98.2%.

The positive control preparations were aliquoted into test tubes and cryopreserved at −20°C or below. The preparations were used within 9 months after preparation (expiration date: one year after preparation), and stored preparations were used within 0.5 h after being thawed. Each preparation was added at a concentration of 1% (vol) to the culture medium in each plate.

5. Cell line

The cell line was CHL/IU, obtained at a passage number of 14 from Dainippon Pharmaceutical Co., Ltd., on May 17, 2005. These cells were derived from the lung of a female newborn Chinese hamster, with 25 chromosomes (mode) (2n = 22) and a cell doubling time of 13.3 h (measured value). This cell line was selected in consideration of the growth rate, chromosomal stability during subcultivation, ease of observation of chromosome specimens, and sensitivity to known mutagens. Cells cryopreserved together with those used in the test were checked for mycoplasma contamination using fluorescent staining, and the results were negative for contamination.

A cell suspension was prepared at a density of 1×10^6 cells/mL using culture medium containing 10% (vol) dimethyl sulfoxide and aliquoted into ampoules (1 mL/ampoule), which were then successively frozen and stored in liquid nitrogen. After being thawed, the cells were seeded in a 75-cm^2 flask and incubated in a CO_2 incubator (MCO-175, Sanyo Electric Co., Ltd.; or MCO-18AIC [UV], Sanyo Electric Biomedical Co., Ltd.) at 5.0% CO_2 and 37.0°C. The cells were subcultured every three or four days. Cells with a passage number of 18 (preliminary test) or 17 (main test) were used in the test.

6. Culture medium

A culture medium was prepared as follows using Eagle MEM.

First, 9.4 g of Eagle MEM (Code 05900, Lot Nos. 62791211 and 628001, containing kanamycin and phenol red, Nissui Pharmaceutical Co., Ltd.) was dissolved in Japanese Pharmacopoeia water for injection (Lot No. 8L88, Otsuka Pharmaceutical Factory, Inc.) to obtain a volume of 1 L. The solution was autoclaved for sterilization, then cooled to room temperature and mixed with a sterilized 7.5% sodium bicarbonate solution (special grade, Lot No. 905X1946, Kanto Chemical Co., Ltd.) to adjust the pH to 7.2 to 7.4. The solution was then mixed with a filter-sterilized L-glutamine solution (special grade, L-glutamine, Lot No. PEH6211, Wako Pure Chemical Industries, Ltd.) to obtain a concentration of 0.292 g/L. Fetal bovine serum

(Lot No. 366272, GIBCO) was added to the mixture to obtain a final concentration of 10%. The fetal bovine serum was inactivated at 56°C for 30 min before use. All procedures following autoclaving were performed aseptically.

7. S9 mix

After purchase from Kikkoman Corporation, S9 mix (Lot No. CAM-605, manufactured on November 20, 2009) was cryopreserved at −80°C or below and used within 6 months after the production date (expiration date: 6 months after production).

To prepare S9 mix, 2.45 mL of cofactor mix was added to 1.05 mL of S9 prepared using liver homogenate from Slc:SD rats (males, 7 weeks of age) intraperitoneally administered with phenobarbital and 5,6-benzoflavone to induce production of drug-metabolizing enzymes. The composition of S9 mix is presented in the following table:

Composition of S9 mix in 1 mL		
S9	(Kikkoman Corporation, Lot No. RAA-605, protein content in S9: 26.23 mg/mL)	0.3 mL
$MgCl_2$	(Wako Pure Chemical Industries, Ltd., Lot No. SDN0075)	5 μmol
KCl	(Wako Pure Chemical Industries, Ltd., Lot No. CDP6106)	33 μmol
G-6-P	(Oriental Yeast Co., Ltd., Lot No. 118904)	5 μmol
NADP	(Oriental Yeast Co., Ltd., Lot No. 045909)	4 μmol
HEPES buffer	(Dojindo Laboratories, Lot No. PE026)	4 μmol
Distilled water		0.1 mL

8. Test methods

8.1. Preliminary test (cell-growth inhibition test)

8.1.1. Test groups

The preliminary test was performed in three test series: short-term treatment without metabolic activation, short-term treatment with metabolic activation, and continuous treatment for 24 h.

The highest dose of the test substance was set at 1870 μg/mL, equivalent to 10 mM (molecular weight: 187.07) according to the test guideline, and 7 lower doses were selected in a twofold dilution series (8 doses in total: 1870, 935, 468, 234, 117, 58.4, 29.2, and 14.6 μg/mL). In addition, a negative control group was included in each test series.

Two plates were used for each group, each marked with an identification number.

8.1.2. Cell seeding

Five milliliters of cell suspension was seeded in a 60-mm plate and incubated in a CO_2 incubator at 5.0% CO_2 and 37.0°C. The cell density in the suspension was 0.4×10^4 cells/mL for short-term treatment without metabolic activation and for continuous treatment for 24 h, and 0.6×10^4 cells/mL for short-term treatment

with metabolic activation.

8.1.3. Treatment with test solutions

a) Short-term treatment without metabolic activation

On the third day from cell seeding, the culture medium in the plate was removed. After the test solution was mixed with fresh medium in a ratio of 300 µL to 2.7 mL in a test tube, 3 mL of the mixture was added to the plate and incubated for 6 h. After completion of incubation, the fluid in the plate was removed, and the remaining cells were rinsed with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffer. Then, 5 mL of fresh medium was added to the plate and incubated for an additional 18 h.

b) Short-term treatment with metabolic activation

On the third day from cell seeding, the culture medium in the plate was removed. After the mixture of S9 mix and fresh medium was mixed with the test solution in a ratio of 0.5 mL:2.2 mL:300 µL in a test tube (final concentration of S9: approximately 5% [vol]), 3 mL of the mixture was added to the plate and incubated for 6 h. After completion of incubation, the fluid in the plate was removed, and the remaining cells were rinsed with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffer. Then, 5 mL of fresh medium was added to the plate and incubated for an additional 18 h.

c) Continuous treatment for 24 h

On the third day from cell seeding, the culture medium in the plate was removed. After the test solution was mixed with fresh medium in a ratio of 500 µL to 4.5 mL in a test tube, 5 mL of the mixture was added to the plate and incubated for an additional 24 h.

8.1.4. Observation for precipitation of the test substance

At the start and end of treatment with the test solution, the plate was macroscopically observed for precipitation of the test substance.

8.1.5. Observation for effects of the test substance on the culture pH

At the start and end of treatment with the test solution, the culture medium in the plate was macroscopically observed for color change. When no color change was found, the test substance was considered to have no effect on the culture pH.

8.1.6. Measurement of the cell growth rate and calculation of the concentration causing 50% inhibition of cell growth (IC_{50})

After completion of incubation, the fluid in the plate was removed, and the remaining cells were rinsed with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffer. The cells were then fixed in 10% formalin for approximately 10 to 15 min and stained with 0.1% (w/v) crystal violet for approximately 10 to 15 min. After staining, the plate was washed with tap water in a water bath and air-dried. The

cell growth rate was measured for each plate using a monolayer culture cell densitometer (MONOCELLATER II, Toyo Sokki Co., Ltd.), with the growth rate in the negative control group defined as 100%. As a decrease in growth rate by 50% or more was not detected in the test substance groups, the concentration causing 50% inhibition of cell growth (IC_{50}) was not calculated.

8.2. Main test (chromosomal aberration test)

8.2.1. Test groups

In the preliminary test, cell growth was not affected by short-term treatment with or without metabolic activation, and was slightly inhibited at high concentration of continuous treatment for 24 h. Accordingly, 1870 μg/mL was selected as the highest dose of each test series, and lower doses were set with a twofold dilution series to select 3 doses in total for short-term treatment with and without metabolic activation, and 4 doses in total for continuous treatment for 24 h.

Four plates were used for each group except the positive control group, including 2 plates for the satellite group to observe the effect of the test substance on cell growth. Two plates were used for the positive control group. Each plate was marked with an identification number.

8.2.2. Cell seeding

The method described in "*8. Test methods, 8.1. Preliminary test, 8.1.2. Cell seeding*" was used.

8.2.3. Treatment with test solutions

a) Short-term treatment without metabolic activation
The method described in "*8. Test methods, 8.1. Preliminary test, 8.1.3. Treatment with test solutions, a) Short-term treatment without metabolic activation*" was used.

b) Short-term treatment with metabolic activation
The method described in "*8. Test methods, 8.1. Preliminary test, 8.1.3. Treatment with test solutions, b) Short-term treatment with metabolic activation*" was used.

c) Continuous treatment for 24 h
The method described in "*8. Test methods, 8.1. Preliminary test, 8.1.3. Treatment with test solutions, c) Continuous treatment for 24 h*" was used.

8.2.4. Observation for precipitation of the test substance

The method described in "*8. Test methods, 8.1. Preliminary test, 8.1.4. Observation for precipitation of the test substance*" was used.

8.2.5. Observation for effects of the test substance on the culture pH

The method described in "*8. Test methods, 8.1. Preliminary test, 8.1.5. Observation for effects of the test substance on the culture pH*" was used.

8.2.6. Measurement of the cell growth rate

The method described in "*8. Test methods, 8.1. Preliminary test, 8.1.6. Measurement of the cell growth rate and calculation of the concentration causing 50% inhibition of cell growth (IC_{50})*" was used. The IC_{50} was not calculated.

8.2.7. Preparation of chromosome specimens

Approximately 2 h before the end of incubation, colcemid (Lot No. 571750, GIBCO) was added to each plate at a final concentration of 0.2 μg/mL. At the end of incubation, the fluid in each plate was collected into respective centrifuge tubes as follows: The plate was first treated with 0.02% EDTA (0.5M EDTA: Lot No. 1390894, GIBCO)-0.25% trypsin (2.5% trypsin: Lot Nos. 633330 and 690264, GIBCO) to detach the cells. The resultant cell suspension was then collected into a centrifuge tube and centrifuged at 1,000 rpm for 5 min. After the supernatant was removed, 0.075 mol/L potassium chloride (Lot No. 810X1990, Kanto Chemical Co., Ltd.) was added to the cells, and the mixture was allowed to stand for 30 min at ordinary temperature with gentle pipetting while the cells swelled. After iced Carnoy's fixative (mixture of methanol and acetic acid in a ratio of 3:1. Methanol, Lot No. 110N1127, Kanto Chemical Co., Ltd.; acetic acid, Lot No. KWF0791, Wako Pure Chemical Industries, Ltd.) was added to fix the cells, the mixture was centrifuged at 1,000 rpm for 5 min. After removing the supernatant, Carnoy's fixative was added to the cells. After these procedures were repeated three times, the cell suspension was dropped onto a glass slide, which was then allowed to air-dry at least overnight. Two chromosome specimens were prepared from each plate.

The slide was stained with 2% Giemsa solution (Giemsa solution, Lot No. KN999, Wako Pure Chemical Industries, Ltd.; instant phosphate buffer (pH 7.2), Lot No. A941, Mitsubishi Chemical Medience Corporation) for 20 min. After being washed with water and air-dried, the slide was sealed with a mounting agent (Malinol: Lot No. 0701201, Muto Pure Chemicals Co., Ltd.).

8.2.8. Observation of chromosome specimens

Prior to observation of specimens, one slide was selected per plate for each dose and blind-coded.

Using a microscope (BX51TF, Olympus Corporation) with a total magnification of 600, 100 metaphase chromosomes were observed in each slide. Chromosomal aberrations were assessed according to the following classification systems. Cells with 25 ± 2 chromosomes were observed for structural aberrations.

a) Structural aberration

- Chromatid break (ctb)
  Distinctive discontinuity (break) in the chromatid; either a discontinuity wider than the chromatid or a break off the major axis of the chromatid

- Chromatid exchange (cte)
  At least two breaks in the chromatid exchanged with each other (combined)
- Chromosome break (csb)
  Break at the same position in a pair of chromatids and judged according to the criteria for chromatid break
- Chromosome exchange (cse)
  Exchange at the same position in a pair of chromatids toward the same direction
- Others
  Other structural aberrations include fragmentation (defined as breaks or gaps affecting almost all metaphase chromosomes with no exchange-type aberration)

b) Gap

Unstained area (no stain) in chromatids or chromosomes with a width less than that of the chromatid

c) Numerical aberration

- Polyploid (poly)
  Triploid, tetraploid, etc., the chromosomal constitution of a cell containing multiples of the normal number of chromosomes (25 ± 2)
- Others
  Other numerical aberrations include endoreduplication (defined as double chromosomes not separated but arranged in parallel), being distinguished from polyploid.

8.2.9.Calculation of observation results

The number of cells with aberrations described below was counted for each plate, and the total sum was calculated for each test group. In addition, the incidence (%) was calculated for the total number of cells with structural aberrations (a cell with more than one structural aberration was counted as one) as well as the total number of cells with numerical aberrations. The incidence (%) was calculated as the percentage of the number of cells with aberrations relative to the total number of cells observed (number of metaphase chromosomes).

a) Structural aberrations

- ctb: number of cells with chromatid breaks
- cte: number of cells with chromatid exchanges
- csb: number of cells with chromosome breaks
- cse: number of cells with chromosome exchanges
- others: number of cells with other structural aberrations
- total: number of cells with any structural aberrations

b) Gap

- gap: number of cells with gaps

c) Numerical aberration

- poly: number of polyploid cells
- others: number of cells with other numerical aberrations
- total: number of cells with any numerical aberrations

9. Evaluation of test results

The results were determined to be positive when the total incidence (%) of structural or numerical aberrations was 10% or more in a dose-dependent manner, or when reproducibility was shown in the results of 5% or more in the confirmatory test. All other results were determined to be negative. Statistical analysis was not used.

As an incidence of aberrations of 5% or more was not detected in any test series, a D_{20} value (the concentration at which 20% of the cells show aberrations) was not calculated.

RESULTS

1. Preliminary test

The results of the cell growth rates are shown in Table 1 and Figure 1, and the results of precipitation of the test substance and effects on the culture pH are shown in Table 1.

No effects were noted in cell growth after short-term treatment with or without metabolic activation. Cell growth was slightly inhibited at doses of 935 and 1870 μg/mL after continuous treatment for 24 h, and the cell growth rates were 64.5% and 65.5%, respectively.

No precipitation of the test substance was noted in any test series.

No effects of the test substance were noted in the culture pH in any test series.

2. Main test

The results of the cell growth rates, precipitation of the test substance, and effects on the culture pH in the main test are shown in Table 2, and the results of clastogenicity evaluation are shown in Tables 3-1 to 3-3.

In the evaluation of effects of the test substance on cell growth in the satellite group, which was performed in parallel with the evaluation of clastogenicity, cell growth was slightly inhibited at 1870 μg/mL only after continuous treatment for 24 h, and the cell growth rate was 63.0%.

No precipitation of the test substance was noted in any test series.

No effects of the test substance were noted in the culture pH in any test series.

Both the incidences of structural and numerous aberrations were less than 5% after short-term treatment without metabolic activation (doses tested: 468 to 1870 μg/mL), short-term treatment with metabolic activation (doses tested: 468 to 1870 μg/mL), and continuous treatment for 24 h (doses tested: 234 to 1870 μg/mL).

The incidences of structural aberrations in the positive control group were 64.0% after short-term treatment without metabolic activation, 39.0% after short-term treatment with metabolic activation, and 64.0% after continuous treatment for 24 h.

DISCUSSION

The clastogenic potential of was evaluated *in vitro* using Chinese hamster lung (CHL/IU) cells.

Based on the results of the preliminary test (cell-growth inhibition test), the highest dose was set at 10 mM, which is the upper limit concentration defined in the test guideline, for each test series, and totally 3 or 4 doses were set for the main test (chromosomal aberration test).

In the main test, both the incidences of structural and numerous aberrations were less than 5% at all doses of all test series, which showed negative results.

In the positive control group, the incidence of structural aberrations of chromosomes was clearly positive in all test series, demonstrating that the cell line used in this study had sufficient sensitivity.

These results indicate that is not clastogenic in cultured mammalian cells under the conditions used in the present test.

ENVIRONMENTAL FACTORS THAT MIGHT HAVE AFFECTED THE RELIABILITY OF THE TEST RESULTS

No environmental factors that might have affected the reliability of the test results were found.

STORAGE OF DOCUMENTS AND MATERIALS

1. Archive facilities and documents and materials to be stored

   The following documents and materials will be stored in the archives of Safety Research Institute for Chemical Compounds Co., Ltd.:

   1. Study protocol and study protocol amendments
   2. Raw data and other documents
   3. Final report
   4. Specimens

2. Storage period

   The above-mentioned documents and materials will be stored for 10 years after the study completion. Their storage thereafter will be decided on agreement with the study sponsor.

NAME AND SEAL OF STUDY DIRECTOR

Name and seal affixed in the original	August 10, 2010
Kohtaro Kawamura, Study Director	Date

Table 1 Effects of on growth rate of CHL/IU with or without metabolic activation (preliminary test) (SR09243)

Growth rate (% to the control)

Group	Concentration (μg/mL)	S9- 6-18 h (Mean)	S9+ 6-18 h (Mean)	S9- 24-0 h (Mean)
Control [a]	–	100 , 100 (100.0)	100 , 100 (100.0)	100 , 100 (100.0)
	14.6	97 , 98 (97.5)	91 , 96 (93.5)	98 , 92 (95.0)
	29.2	99 , 99 (99.0)	92 , 96 (94.0)	97 , 91 (94.0)
	58.4	92 , 86 (89.0)	97 , 90 (93.5)	99 , 87 (93.0)
	117	85 , 98 (91.5)	87 , 92 (89.5)	88 , 84 (86.0)
	234	95 , 90 (92.5)	97 , 92 (94.5)	94 , 91 (92.5)
	468	91 , 92 (91.5)	92 , 94 (93.0)	88 , 77 (82.5)
	935	83 , 86 (84.5)	89 , 96 (92.5)	70 , 59 (64.5)
	1870	78 , 82 (80.0)	88 , 106 (97.0)	78 , 53 (65.5)
IC_{50} (μg/mL)		-	-	-

a : Water for injection (Japanese pharmacopoeia)
Precipitation and change of pH in culture medium were not observed.
The figure in parentheses represents mean value of two plates.
-: Blank

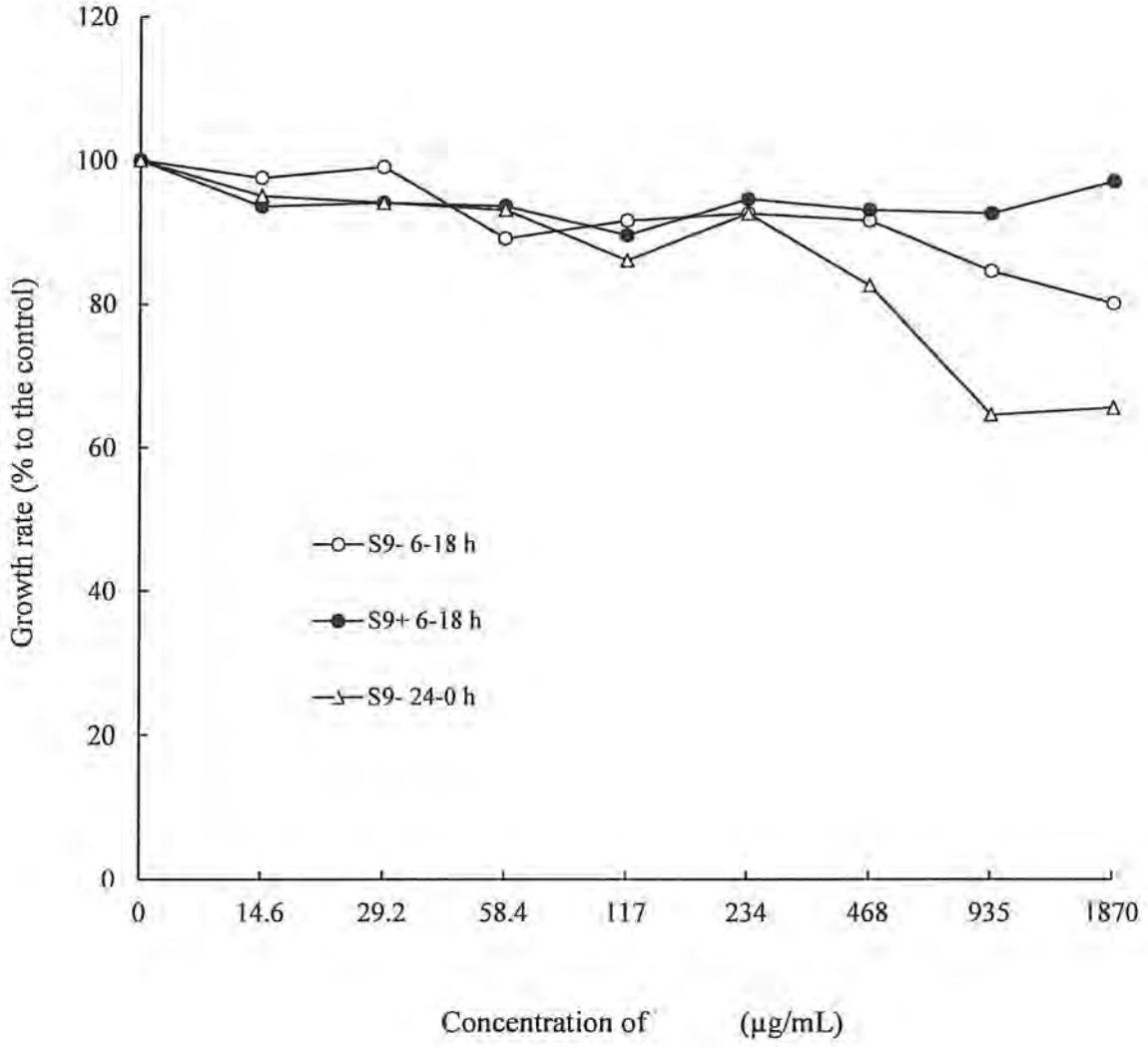

Figure 1 Effects of on growth rate of CHL/IU with or without metabolic activation (preliminary test) (SR09243)

Each point represents mean value (n=2).

Table 2 Effects of on growth rate of CHL/IU with or without metabolic activation (chromosomal aberration test) (SR09243)

Growth rate (% to the control)

Group	Concentration (μg/mL)	S9- 6-18 h (Mean)	S9+ 6-18 h (Mean)	S9- 24-0 h (Mean)
Control [a]	–	100 , 100 (100.0)	100 , 100 (100.0)	100 , 100 (100.0)
	234	-	-	92 , 115 (103.5)
	468	100 , 102 (101.0)	97 , 104 (100.5)	96 , 101 (98.5)
	935	99 , 106 (102.5)	105 , 104 (104.5)	78 , 85 (81.5)
	1870	92 , 90 (91.0)	108 , 105 (106.5)	62 , 64 (63.0)

a : Water for injection (Japanese pharmacopoeia)
Precipitation and change of pH in culture medium were not observed.
The figure in parentheses represents mean value of two plates.
-: Blank

Table 3-1 Results of the chromosomal aberration test of (6 hours treatment without metabolic activation) (SR09243)

Time schedule [a] (hours)	S9	Group	Concentration (μg/mL)	Growth rate (%)	Number of metaphase observed	Structural aberrations						Gap	Numerical aberrations			Judgment [c]
						ctb	cte	csb	cse	others	total (%)		poly	others	total (%)	
6-18	–	Control [b]	—	100.0	100	0	1	0	0	0	1	0	0	0	0	–
					100	0	0	0	0	0	0	0	1	0	1	
					200	0	1	0	0	0	1 (0.5)	0	1	0	1 (0.5)	
			468	101.0	100	0	0	0	0	0	0	0	0	0	0	–
					100	0	0	0	0	0	0	0	0	0	0	
					200	0	0	0	0	0	0 (0.0)	0	0	0	0 (0.0)	
			935	102.5	100	0	0	0	0	0	0	0	0	0	0	
					100	0	0	0	0	0	0	0	1	0	1	
					200	0	0	0	0	0	0 (0.0)	0	1	0	1 (0.5)	
			1870	91.0	100	0	0	0	0	0	0	0	0	0	0	
					100	0	0	0	0	0	0	0	0	0	0	
					200	0	0	0	0	0	0 (0.0)	0	0	0	0 (0.0)	
		Mitomycin C	0.1		100	12	57	0	2	0	66	0	0	0	0	+
					100	13	55	0	1	0	62	1	0	0	0	
					200	25	112	0	3	0	128 (64.0)	1	0	0	0 (0.0)	

ctb, chromatid break cte, chromatid exchange csb, chromosome break cse, chromosome exchange poly, polyploid

a : Time schedule ; treatment time-recovery time

b : Water for injection (Japanese pharmacopoeia)

c : Judgment was made according to the total (%) of structural aberrations and numerical aberrations ; –, negative +, positive

Table 3-2 Results of the chromosomal aberration test of (6 hours treatment with metabolic activation) (SR09243)

Time schedule [a] (hours)	S9	Group	Concentration (μg/mL)	Growth rate (%)	Number of metaphase observed	Structural aberrations						Gap	Numerical aberrations			Judgment [c]
						ctb	cte	csb	cse	others	total (%)		poly	others	total (%)	
6-18	+	Control [b]	—	100.0	100	0	0	0	0	0	0	0	0	0	0	−
					100	0	0	0	0	0	0	0	0	0	0	
					200	0	0	0	0	0	0 (0.0)	0	0	0	0 (0.0)	
			468	100.5	100	0	0	0	0	0	0	0	0	0	0	−
					100	0	0	0	0	0	0	0	0	0	0	
					200	0	0	0	0	0	0 (0.0)	0	0	0	0 (0.0)	
			935	104.5	100	0	0	0	0	0	0	0	0	0	0	
					100	0	0	0	0	0	0	0	0	0	0	
					200	0	0	0	0	0	0 (0.0)	0	0	0	0 (0.0)	
			1870	106.5	100	1	1	0	0	0	1	0	0	0	0	
					100	0	0	0	0	0	0	0	1	0	1	
					200	1	1	0	0	0	1 (0.5)	0	1	0	1 (0.5)	
		3,4-Benzopyrene	10		100	7	32	0	0	0	37	0	0	0	0	+
					100	12	33	2	0	0	41	0	0	0	0	
					200	19	65	2	0	0	78 (39.0)	0	0	0	0 (0.0)	

ctb, chromatid break cte, chromatid exchange csb, chromosome break cse, chromosome exchange poly, polyploid

a : Time schedule ; treatment time-recovery time

b : Water for injection (Japanese pharmacopoeia)

c : Judgment was made according to the total (%) of structural aberrations and numerical aberrations ; −, negative +, positive

Table 3-3 Results of the chromosomal aberration test of (24 hours treatment without metabolic activation) (SR09243)

Time schedule [a] (hours)	S9	Group	Concentration (μg/mL)	Growth rate (%)	Number of metaphase observed	Structural aberrations						Gap	Numerical aberrations			Judgment [c]
						ctb	cte	csb	cse	others	total (%)		poly	others	total (%)	
24-0	−	Control [b]	—	100.0	100	0	0	0	0	0	0	0	0	0	0	−
					100	0	0	0	0	0	0	0	0	0	0	
					200	0	0	0	0	0	0 (0.0)	0	0	0	0 (0.0)	
			234	103.5	100	0	0	0	0	0	0	0	0	0	0	−
					100	1	0	0	0	0	1	0	1	0	1	
					200	1	0	0	0	0	1 (0.5)	0	1	0	1 (0.5)	
			468	98.5	100	0	0	0	0	0	0	0	0	0	0	
					100	0	0	0	0	0	0	0	1	0	1	
					200	0	0	0	0	0	0 (0.0)	0	1	0	1 (0.5)	
			935	81.5	100	0	0	0	0	0	0	0	2	0	2	
					100	0	0	0	0	0	0	0	0	0	0	
					200	0	0	0	0	0	0 (0.0)	0	2	0	2 (1.0)	
			1870	63.0	100	0	1	0	0	0	1	0	0	1	1	
					100	0	1	0	0	0	1	0	0	1	1	
					200	0	2	0	0	0	2 (1.0)	0	0	2	2 (1.0)	
		Mitomycin C	0.05		100	10	59	0	1	0	67	0	0	0	0	+
					100	6	58	0	0	0	61	0	0	0	0	
					200	16	117	0	1	0	128 (64.0)	0	0	0	0 (0.0)	

ctb, chromatid break cte, chromatid exchange csb, chromosome break cse, chromosome exchange poly, polyploid

a : Time schedule ; treatment time-recovery time

b : Water for injection (Japanese pharmacopoeia)

c : Judgment was made according to the total (%) of structural aberrations and numerical aberrations ; −, negative +, positive

January 14, 2010

Analysis table

1. Name of article

2. Analysis values

Items	Analysis values	Notes

August 1, 2010

Analysis table

1. Name of article

2. Analysis values

Items	Analysis values	Notes